Biochemical Studies of the Excitable Membrane of *Paramecium tetraurelia*

VI. Endogenous Protein Substrates for In Vitro and In Vivo Phosphorylation in Cilia and Ciliary Membranes

ROBERT MASON LEWIS and DAVID L. NELSON
Department of Biochemistry, University of Wisconsin –Madison, Madison, Wisconsin 53706

ABSTRACT   The endogenous protein kinases of isolated *Paramecium tetraurelia* cilia phosphorylated ~30 ciliary polypeptides in vitro. Labeling with [γ-^{32}P]ATP was not proportional to the amount of each protein in cilia; some minor polypeptides (e.g., 67,000 and 180,000 mol wt) were more heavily labeled than some major polypeptides. Certain of the endogenous substrates for protein kinase were localized in the ciliary membrane (130,000, 86,000, 67,000, and 45,000 mol wt); others were found in axonemes or in both fractions. With cilia from bacterized cultures in the undefined Cerophyl medium, the labeling of specific endogenous phosphate acceptors was altered by pH, cyclic AMP, and cyclic GMP, but the labeling pattern was not affected by the presence of Na$^+$ or K$^+$ (15 mM), Ba^{++} (5 mM), Ca^{++} (10^{-5} or 10^{-4} M), or EGTA. Very similar results were obtained with cilia from cells grown axenically in a semidefined medium; the molecular weights and the extent of phosphorylation of the phosphopolypeptides were comparable to those of cilia from bacterized Cerophyl cultures, although no significant cyclic nucleotide effects were observed in the axenic cilia. Most of the phosphopolypeptides labeled in vitro also turned over rapidly in vitro. The phosphoprotein phosphatase responsible for turnover was partially inhibited by 5 mM NaF.

The pattern of ciliary polypeptides labeled in vivo was similar to that observed in the in vitro experiments, although the relative intensities of labeling differed. Six behavioral mutants of *Paramecium*, known to have defects in the excitable membrane that regulates the ciliary beat, showed normal patterns of ciliary protein phosphorylation in vitro, with and without added cyclic nucleotides, at both pH 6.0 and pH 8.0. The mutants also had apparently normal phosphoprotein phosphatase. The Paranoiac A mutant, however, showed a reduction in cyclic GMP–stimulated protein kinase activity.

The excitable ciliary membrane of *Paramecium* regulates the direction of the ciliary beat, and thereby the direction of swimming of this protozoan. Any of several types of stimuli (chemical, physical, or thermal) elicit an "avoiding response": membrane depolarization causes voltage-sensitive Ca^{++} channels in the ciliary membrane to open, and the consequent influx of Ca^{++} triggers ciliary reversal and backward swimming (1, 2). The initial events of excitation occur very rapidly (in milliseconds), but other associated changes in membrane properties or swimming behavior are very much slower. Some examples are: Saimi and Kung (3) have recently described a

slow, inward, Ca^{++}-induced Na$^+$ current initiated by excitation; backward swimming after stimulation with high concentrations of K$^+$ ceases after a few seconds; and a longer-term "adaptation" to K$^+$ stimulation, in which cells become insensitive to a second stimulus such as Ba^{++} or high temperature, that requires hours (4).

There is little available information concerning the mechanisms that regulate these slow, reversible changes in the stimulus-response pathway of *Paramecium*, but there is evidence in other systems that the reversible phosphorylation of proteins in a pathway modifies their activity and regulates the pathway

in which they function. In several types of excitable cells, membrane protein phosphorylation or dephosphorylation occurs in association with stimulation (5, 6). Cyclic AMP, which stimulates many protein kinases, stimulates flagellar motility of mammalian sperm (7–9) and inhibits the motility and regeneration of *Chlamydomonas* flagella (10) and the regeneration of *Tetrahymena* cilia (11). A cyclic AMP–dependent protein kinase and three cyclic GMP–dependent protein kinases have been isolated from the membrane or soluble fraction of *Tetrahymena pyriformis* cilia (12), and isolated axonemes of *Tetrahymena* cilia contain a cyclic AMP–dependent protein kinase (13).

We (14) have shown that unfractionated cilia of *Paramecium* contain at least two distinguishable protein kinase activities, one sensitive to cyclic nucleotides, and Schultz and Jantzen (15) have chromatographically resolved three protein kinases (one dependent on cyclic GMP, two dependent upon cyclic AMP) from *Paramecium* cilia. *Paramecium* cilia also contain a guanylate cyclase activity that is inhibited by Ca^{++} (16). Haga and Kung (University of Wisconsin–Madison, personal communication) have recently found that the microinjection of cyclic AMP and cyclic GMP into paramecia modifies their swimming activity. It therefore seems possible that cyclic nucleotide–dependent protein kinases are involved in the regulation of ciliary motility in *Paramecium*, either acting on membrane proteins and thus affecting electrical properties of the membrane, or acting directly upon axonemal proteins to alter the ciliary beat.

If protein kinases play a role in the regulation of ciliary beat (in either excitation or adaptation) in *Paramecium*, the identification of specific phosphorylated proteins may provide a clue to the mechanisms of regulation. We have therefore investigated the endogenous protein substrates for phosphorylation in vitro in isolated cilia, and in cilia isolated after phosphorylation in vivo. We have also compared the ciliary phosphoproteins of wild-type cells with those of several mutant strains defective in the regulation of swimming behavior, seeking specific proteins the phosphorylation of which may be altered in the mutants.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was synthesized by the method of Schendel and Wells (17). Cerophyl powder was obtained from Cerophyl Laboratories, Inc. (Kansas City, Mo.). Pronase, ATP, Tris, MOPS (morpholinopropanesulfonic acid), 3-isobutyl-1-methylxanthine, caffeine, indomethacin, cyclic AMP (adenosine 3':5'-cyclic monophosphoric acid) and cyclic GMP (guanosine 3':5'-cyclic monophosphoric acid) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Acrylamide and BIS (*N,N'*-methylene-*bis*-acrylamide) were obtained from Bio-Rad Laboratories, Inc. (Richmond, Calif.). SDS was recrystallized twice from the "specially pure" grade obtained from BDH Biochemicals, Ltd. (Poole, England). Nutrient broth was obtained from Difco Laboratories (Detroit, Mich.).

Stocks and Cultures

Paramecium tetraurelia, wild-type stock 51S and mutants Pawn A (d4-94), Pawn B (d4-95), fna^P (d4-149), Paranoiac C (d4-150), fna (d4-91) and Paranoiac A (d4-90) (supplied by Dr. Ching Kung, University of Wisconsin–Madison), was grown at 28°C in phosphate-buffered Cerophyl medium bacterized with *Enterobacter aerogenes* as previously described (18). To eliminate the trivial possibility that the results were attributable to small amounts of contaminating bacteria, we also performed the experiments with cells grown axenically in the Soldo crude medium (19).

In Vitro Protein Phosphorylation of Cilia and Subciliary Fractions

Cilia and subciliary fractions (membranes, axonemes, and incompletely demembranated cilia) were isolated as previously described (18) with slight modifications (14). Bacteria remaining in the cilia preparations either were counted directly on a Petroff-Hausser bacteria counter, or aliquots were plated on Cerophyl agar plates and the colonies that appeared after an overnight incubation at 28°C were counted. These values were used to estimate that the bacteria contributed ~0.001% by weight of the total protein in each sample of cilia.

In vitro substrates of the pH 6.0 protein kinase (14) were determined by suspending 30–300 μg of cilia, membranes, axonemes, or incompletely demembranated cilia in 0.1 ml of 10 mM MOPS, 1.5 mM Tris (pH 6.0), 4 mM MgCl$_2$, and any additional substrates or reagents required for the assay. After a 15-min preincubation, the reaction was initiated by the addition of 1–2 μCi of [γ-^{32}P]ATP adjusted to a specific activity of 0.1–0.2 μCi/nmol. The final concentration of ATP was 0.1 mM. After a 15-s incubation at 28°C (unless otherwise noted), the reaction was terminated by the addition of 0.05 ml of SDS sample buffer (7% [wt/vol] SDS, 25% [vol/vol] glycerol, 188 mM Tris, 0.01% [wt/vol] bromophenol blue, pH 6.8). The sample was heated at 100°C for 5 min, 7.5 μl of 2-mercaptoethanol were added, and the sample was stored (if necessary) at −20°C. In vitro substrates of the pH 8.0 protein kinase (14) were determined in a similar manner, except that 10 mM MOPS, 17.5 mM Tris (pH 8.0), and 10 mM MgCl$_2$ were used.

Protein quantities were measured by the method of Lowry et al. (20), using bovine serum albumin as a standard.

In Vivo Labeling of Ciliary Phosphoproteins

The phosphoproteins. of *P. tetraurelia* were labeled in vivo by growing the cells in Cerophyl medium containing *Enterobacter aerogenes* labeled uniformly with ^{32}P. The bacteria were first grown to stationary phase in a medium containing 5% (vol/vol) low-phosphate nutrient broth concentrate (21), 100 mM MOPS, 10 mM KCl, 10 mM MgCl$_2$, 10 mM (NH$_4$)$_2$SO$_4$, pH 6.8 and 20 μCi/ml carrier-free ^{32}P$_i$. Cilia were isolated in the presence of 5 mM NaF, suspended at 3 mg/ml in 0.1 ml of 10 mM MOPS, 5.5 mM Tris (pH 7.0), 5 mM NaF, and prepared for gel electrophoresis as described above.

SDS Gel Electrophoresis and Autoradiography

The proteins were separated on discontinuous SDS polyacrylamide slab gels composed of a 7.5–15% acrylamide gradient running gel and a 3% stacking gel (22–24). Gels were stacked at 50 V and run at 100 V constant voltage for 4.5–5 h with tapwater cooling. The gel was stopped when a cytochrome c marker, which was visible during the run, was ~1 cm from the bottom of the gel. Gels were stained in 0.1% Coomassie Brilliant Blue R250 dissolved in ethanol:H$_2$O: acetic acid (9:9:2), destained in 7.5% acetic acid, 3% glycerol, and dried on filter paper. Molecular weight standards were myosin heavy chain (220,000 mol wt) (kindly provided by Dr. M. Greaser, University of Wisconsin–Madison), conalbumin (86,000), bovine serum albumin (68,000), ovalbumin (43,000), DNase I (34,000), α-chymotrypsinogen A (26,000), myoglobin (17,000), and cytochrome c (13,000). All standards (except myosin) were obtained from Sigma Chemical Co.

The dried gels were exposed to Kodak XR-5 x-ray film for 7 d at room temperature. In some cases a Cronex Lightning-Plus intensifying screen (DuPont (Co., Wilmington, Del.) was used and the gels were exposed at −70°C for 7 d. Known amounts of [γ-^{32}P]ATP were spotted on filter paper and exposed simultaneously with the gels. The autoradiographs of these standards and the gel were scanned with either a Joyce-Loebl densitometer (Joyce, Loebl and Co., Ltd. Gateshead-on-Tyne, England) or a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Chicago, Ill.). The standards and gel samples were within the range in which optical density was linear with radioactivity. The areas of the standard peaks were then used to estimate the number of counts in each phosphopolypeptide based on the area of each of the peaks from the autoradiograph of the gel. The number of moles of phosphate in each band was then calculated from the specific activity of the [γ-^{32}P]ATP used in the experiment. The amount of protein in each band was estimated by scanning the Coomassie staining pattern of the gel and determining the percentage of the total protein represented by each band. These two values were then used to estimate the moles of phosphate incorporated per mole of protein for each phosphopolypeptide. (For this estimate of stoichiometry, autoradiograms, not fluorograms, were used.)

Each of the autoradiographs shown is from a separate preparation of cilia (or subciliary fractions) that was divided into equal aliquots, treated identically (except for the variables described in the figure legends and text), and run on the same gel. A typical autoradiograph from each experiment is shown. The quantitative data are from all of the experiments done for each variable and are expressed as: mean ± standard deviation, *n* being the number of separate
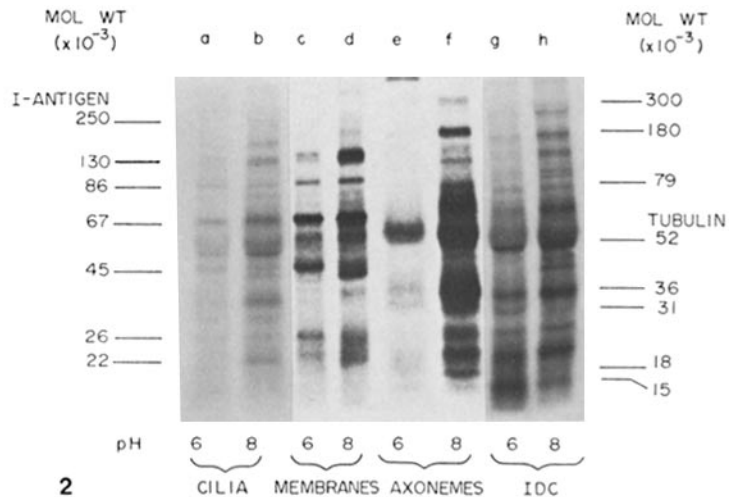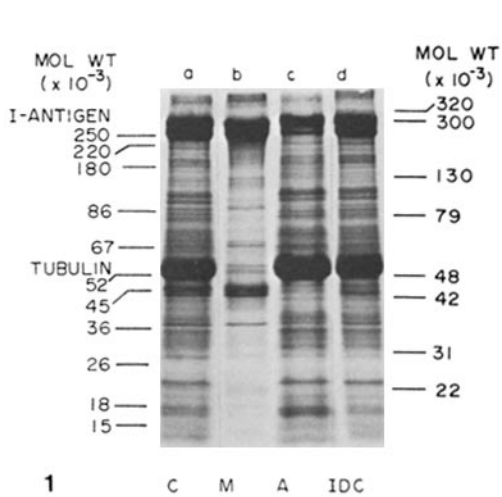
FIGURE 1 Coomassie Blue–staining pattern of cilia and subciliary fractions. (a) Whole cilia (C), 100 μg; (b) ciliary membranes (M), 100 μg; (c) axonemes (A), 45 μg; (d) incompletely demembranated cilia (IDC), 100 μg. I-antigen, immobilization antigen. FIGURE 2 In vitro phosphoproteins of cilia and subciliary fractions. Subciliary fractions were labeled in vitro for 15 s. The autoradiographs for the membranes and axonemes were from the same gel as the other samples, but were exposed simultaneously with a fluorescent screen to increase the intensity of the bands. (a) Cilia, pH 6.0, 100 μg; (b) cilia, pH 8.0, 100 μg; (c) membranes, pH 6.0, 100 μg; (d) membranes, pH 8.0, 100 μg; (e) axonemes, pH 6.0, 45 μg; (f) axonemes, pH 8.0, 45 μg; (g) incompletely demembranated cilia (IDC), pH 6.0, 100 μg; (h) incompletely demembranated cilia (IDC), pH 8.0, 100 μg.

experiments. Reproducible differences in phosphate incorporation of 50% or more between the control and variable samples within each experiment were considered to be significant.

RESULTS

In Vitro Protein Phosphorylation in Cilia and Subciliary Fractions

At least 15 polypeptides in the cilia were labeled in all experiments by the endogenous ciliary protein kinases. Up to 15 other minor phosphopolypeptides were detected less frequently. (Over 70 individual preparations of wild-type cilia were used.) The intensity of each band of the autoradiograph was not directly proportional to the intensity of staining with Coomassie. For example, the immobilization antigen, which (after tubulin) is the most prominent polypeptide in cilia (18) (Fig. 1), was only slightly labeled (Fig. 2). Some relatively minor polypeptides were highly labeled with ^{32}P; for example, 5–10% of the 67,000 and 180,000 mol wt polypeptides were phosphorylated within the first 15 s of the assay (Figs. 1 and 2). Although only 1% of the tubulin molecules were labeled in 15 s, the autoradiograph band corresponding to tubulin was intense because tubulin is the major polypeptide present in cilia (18). If the cilia were treated with pronase (0.5 U/ml, 30 min, 37°C), then all Coomassie-stained bands and all autoradiograph bands were eliminated (two separate experiments, data not shown).

Although similar substrates were labeled at both pH 8.0 and pH 6.0, the overall intensity of labeling was greater at pH 8.0 than at pH 6.0 for the cilia, membranes, axonemes, and incompletely demembranated cilia when equal aliquots of each fraction were labeled at pH 8.0 or pH 6.0 and equal amounts of protein were run side-by-side on a gel and simultaneously autoradiographed (Fig. 2). Very little label was incorporated into the axonemes at pH 6.0 (Fig. 2 e). The relative degree of phosphorylation of specific proteins was also different at pH 8.0 and pH 6.0 (Figs. 2 and 3). These differences were qualitatively reproducible in a total of 10 individual cilia preparations in which aliquots of the same sample were labeled at pH
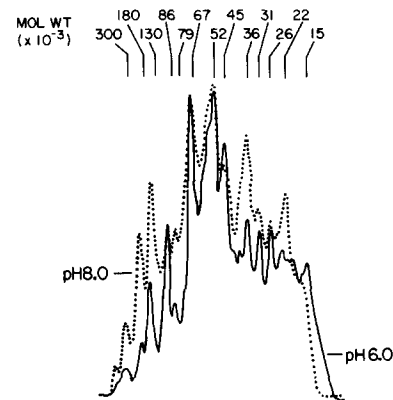

FIGURE 3 Effects of pH on in vitro phosphorylation in cilia. Autoradiographs of cilia labeled at pH 8.0 (dotted line, from Fig. 2 b) and at pH 6.0 (solid line, from Fig. 2 a) were scanned on a densitometer. The band at 86,000 mol wt was chosen as an arbitrary standard, and the corresponding peak was adjusted to the same height for each scan.

8.0 and pH 6.0. Quantitative assessment of the degree of specificity was difficult because of slight, independent variations in the amount of labeling of the individual polypeptides from experiment to experiment, the number of bands which varied, and the overall greater labeling at pH 8.0. Quantitative comparisons will require purification of the kinases and substrates.

The cilia were fractionated into membranes, axonemes, and incompletely demembranated cilia (18). The individual fractions were then labeled to indicate which of the membrane proteins were phosphorylated by the membrane kinases and which of the axonemal proteins were labeled by the axonemal kinases. Phosphopolypeptides of 130,000, 86,000, 67,000, and 45,000 mol wt partitioned consistently with the membranes (Fig. 2 c and d and Fig. 4), whereas phosphopolypeptides of 300,000, 180,000, 52,000 (tubulin), 36,000, 31,000, 18,000, and 15,000 mol wt partitioned consistently with the axonemes (Fig. 2 e and f and Fig. 4). Several phosphopolypeptides (79,000,
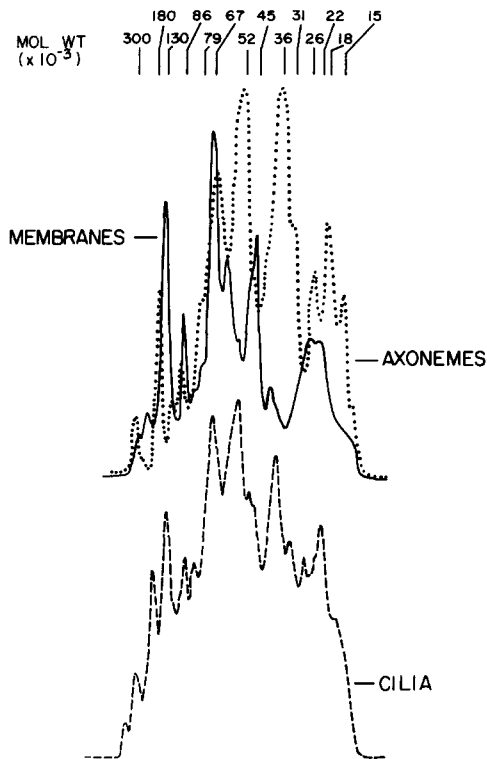
MOL. WT
(x 10⁻³)   300 /130/ 79/ 52 / 36 /26/ 18/
           180 86 67 45 31 22 15

MEMBRANES —

— AXONEMES

—CILIA

26,000, and 22,000 mol wt) were found in both the membrane and axoneme fractions (Fig. 2 c–f and Fig. 4) and may represent structures that link the membranes to the axonemes (25). These results were qualitatively reproducible in six separate experiments. The other phosphoproteins were too faint on the autoradiographs to allow accurate determination of their location. Some phosphoproteins may have been lost or dephosphorylated during fractionation of the cilia.

Ion Effects on In Vitro Phosphorylation

Neither Ca^{2+} at concentrations of 10^{-5} or 10^{-4} M (added as a $CaCl_2$/EGTA buffer) nor 0.1 mM EGTA alone had any effect on the phosphorylation of the endogenous protein substrates at pH 6.0 or pH 8.0 (data from 10 experiments not shown). No effect on protein phosphorylation was observed with the addition of 15 mM KCl (10 experiments), 15 mM NaCl (two experiments), or 5 mM $BaCl_2$ (five experiments) (data not shown).

Cyclic Nucleotide–stimulated Phosphorylation

The effects of cyclic GMP and cyclic AMP on the in vitro phosphorylation of ciliary polypeptides were examined. To inhibit a cyclic nucleotide phosphodiesterase in this preparation (R. M. Lewis and D. L. Nelson, unpublished observations), we added 1 mM 3-isobutyl-1-methylxanthine and 1 mM caf-

feine whenever cyclic nucleotides were added. At pH 8.0, 10^{-5} M cyclic GMP specifically stimulated the phosphorylation of at least three polypeptides (320,000, 45,000, and 31,000 mol wt) (Fig. 5 b and Table I [a]). The phosphorylation of at least two other polypeptides (52,000 [tubulin] and 48,000 mol wt) was also somewhat enhanced by cyclic GMP in some of the experiments. Similar, but slightly less pronounced effects as compared with the cyclic GMP effects in the same experiment, were observed with cyclic AMP (Fig. 5 c and Table I [d]).

It has been reported that indomethacin inhibits cyclic AMP-dependent protein kinases from rabbit (26) and rat (27) ileal mucosa, but in some tissues it stimulates activity (27) and may actually have a nonspecific effect (28). Indomethacin caused only a slight reduction in the stimulation by cyclic GMP in *Paramecium* cilia at pH 8.0 (Fig. 5 b, e, and f). Indomethacin was somewhat more effective against cyclic AMP–stimulated phosphorylation; phosphorylation of the 320,00 mol wt polypeptide was almost completely blocked by as little as 10^{-7} M indomethacin (Fig. 5 g and h). If no cyclic nucleotides were present, 10^{-6} M indomethacin had no effect (Fig. 5 d). Qualitatively similar results were obtained in three separate experiments.

The effect of cyclic GMP or cyclic AMP on phosphorylation was slightly less pronounced at pH 6.0. Phosphorylation of the 45,000 and 31,000 mol wt polypeptides was stimulated by 10^{-5} M cyclic GMP or 10^{-5} cyclic AMP (Fig. 6 and Table I [a and d]). The addition of 5 mM NaF to inhibit phosphoprotein phosphatase activity failed to reveal any stimulation by cyclic nucleotides that would have been missed as a result of rapid dephosphorylation (data from two experiments not shown).
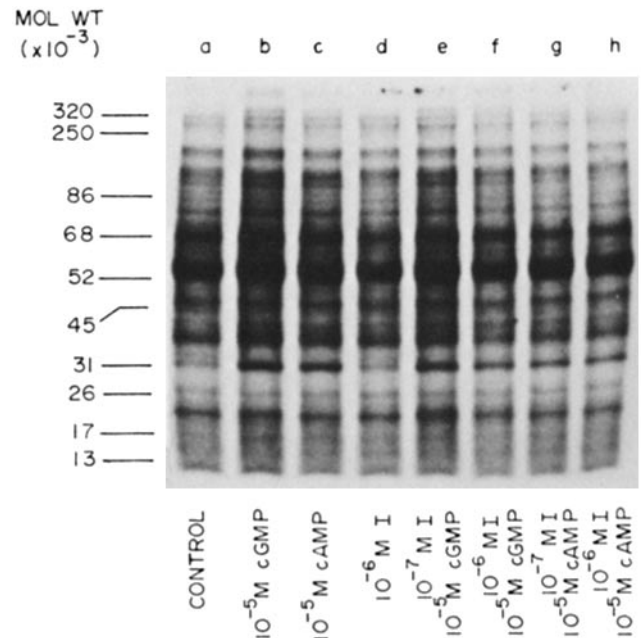
MOL WT
(x10⁻³)   a   b   c   d   e   f   g   h

320 —
250 —
86 —
68 —
52 —
45 —
31 —
26 —
17 —
13 —

CONTROL
10^{-5} M cGMP
10^{-5} M cAMP
10^{-6} M I
10^{-7} M I
10^{-5} M cGMP
10^{-6} M I
10^{-5} M cGMP
10^{-7} M I
10^{-5} M cAMP
10^{-6} M I
10^{-5} M cAMP

TABLE I

Effects of Cyclic GMP and Cyclic AMP on In Vitro Phosphorylation in Wild-type and Paranoiac A (d4-90) Cilia

Molecular weight	pH	% Activity with Cyclic GMP			% Activity with Cyclic AMP		
		(a) Wild type	(b) Paranoiac A	(c) Significance	(d) Wild type	(e) Paranoiac A	(f) Significance
320,000	8.0	310 ± 91, $n = 4$	150 ± 50, $n = 3$	$0.010 < P < 0.025$	280 ± 76, $n = 3$	160 ± 13, $n = 3$	$0.010 < P < 0.025$
45,000	8.0	240 ± 80, $n = 7$	250 ± 79, $n = 3$	NS	260 ± 83, $n = 3$	270 ± 200, $n = 3$	NS
31,000	8.0	330 ± 88, $n = 7$	130 ± 79, $n = 3$	$0.001 < P < 0.005$	230 ± 90, $n = 3$	150 ± 110, $n = 3$	$0.10 < P < 0.25$
45,000	6.0	150 ± 47, $n = 3$	180 ± 38, $n = 3$	NS	160 ± 54, $n = 3$	130 ± 30, $n = 3$	NS
31,000	6.0	210 ± 52, $n = 3$	230 ± 140, $n = 3$	NS	220 ± 110, $n = 3$	190 ± 120, $n = 3$	NS

Effects of cyclic GMP and cyclic AMP on in vitro phosphorylation in wild-type and Paranoiac A (d4-90) cilia. Autoradiographs of equal aliquots of cilia labeled in the presence and absence of cyclic nucleotides were scanned on a densitometer and the areas of the peaks were compared. % Activity = (area of peak with cyclic nucleotide)/(area of peak without cyclic nucleotide × 100%). The data from each experiment were combined and expressed in the table as: mean ± standard deviation, n = number of experiments. (Only four values for percent activity with cyclic GMP for the 320,000 mol wt polypeptide at pH 8.0 were given because this band was not well resolved in the other three experiments.) The Significance indicates the degree of probability that the values for wild type and Paranoiac A are significantly different and is derived from Student's t test and expressed as P. NS, not significant (P > 0.25).
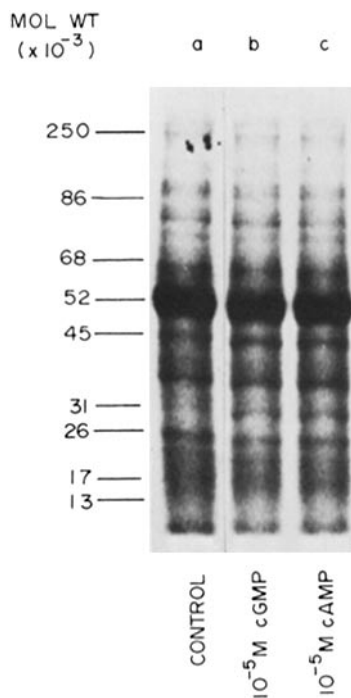

FIGURE 6 Effects of cyclic GMP and cyclic AMP on in vitro phosphorylation at pH 6.0. Whole cilia (100 µg) were labeled in vitro for 15 s. In all cases where cyclic nucleotides were added, 1 mM 3-isobutyl-1-methylxanthine and 1 mM caffeine were also added. A fluorescent screen was used to intensify the image. (a) Control; (b) 10^{-5} M cyclic GMP; (c) 10^{-5} M cyclic AMP.

Phosphoprotein Phosphatase in Cilia

Paramecium cilia contain a phosphoprotein phosphatase that is partially inhibited by NaF (14). Dephosphorylation of the individual phosphopolypeptides by this enzyme was detected by incubating the cilia either with or without NaF and determining the degree of phosphorylation after successive periods of time. The amount of label incorporated in the absence of NaF is a measure of the relative rates of phosphorylation and dephosphorylation, because both the protein kinase and phosphatase are active throughout the assay. All of the detectable phosphoproteins were phosphorylated within 15 s and showed only a slight increase in labeling after longer periods of incubation at pH 8.0 (Fig. 7). NaF had little effect at 15 s (Fig. 7 b and c), but by 15 min in the absence of NaF the amount of incorporated phosphate had decreased (Fig. 7 h). These data
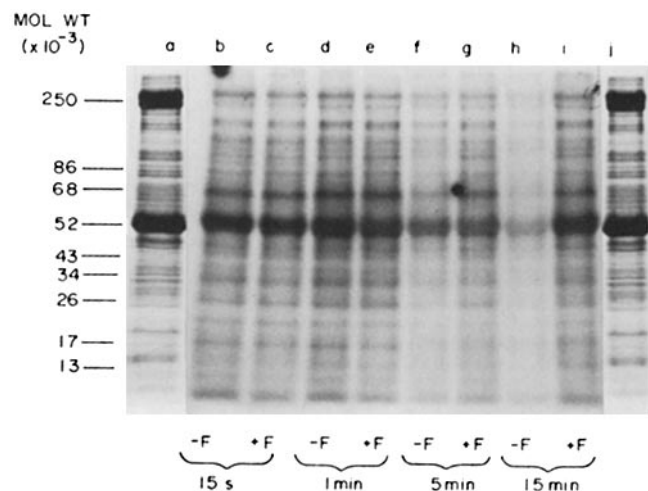

FIGURE 7 Phosphoprotein phosphatase in cilia at pH 8.0. Whole cilia (100 µg) were labeled in vitro. (a) Coomassie stain pattern corresponding to autoradiograph in b. (b-i) Autoradiographs: (b) 15 s; (c) 5 mM NaF, 15 s; (d) 1 min; (e) 5 mM NaF, 1 min; (f) 5 min; (g) 5 mM NaF, 5 min; (h) 15 min. (i) 5 mM NaF, 15 min. (j) Coomassie stain pattern corresponding to autoradiograph in h. The autoradiograph shown is from one of seven experiments with similar results.

imply that after 15 min of incubation the rate of dephosphorylation exceeded the rate of phosphorylation. This effect may have resulted from stimulation of phosphoprotein phosphatase by the increase in phosphate bound to protein (catalyzed by the protein kinase), a decrease in protein kinase activity because of depletion of ATP, or a combination of both. No change in the Coomassie Blue–staining pattern was observed throughout the time-course (Fig. 7 a and j).

Similar results were obtained at pH 6.0, although at least three polypeptides (250,000, 48,000, and 22,000 mol wt) exhibited increased incorporation of phosphate up to 15 min even in the absence of NaF (data not shown).

In Vitro Protein Phosphorylation in Cilia from Axenically Grown Cells

Cilia from cells grown axenically in the Soldo Crude Medium (19) yielded results that were similar, but not identical, to those obtained with cilia from cells grown in bacterized Cerophyl. The in vitro–labeled phosphoproteins displayed a similar overall autoradiograph pattern (Fig. 8 a and e), and phospho-

protein phosphatase was active at both pH 6.0 and pH 8.0 (Fig. 8 *b* and *f*). No significant response to cyclic GMP and cyclic AMP, however, could be detected (Fig. 8 *c*, *d*, *g*, and *h*).

In Vivo Ciliary Phosphoproteins

Cilia from paramecia grown on ^{32}P-labeled *E. aerogenes* were studied to determine which of the phosphoproteins labeled in vitro also occurred in vivo. The molecular weights of the phosphopolypeptides labeled in vivo matched the molecular weights of the polypeptides phosphorylated in vitro (Fig. 9). The most striking difference between the in vivo and in vitro labeling patterns appeared in the relative intensities of the individual bands. The bands labeled most intensely in vivo had molecular weights of 250,000 (I-antigen), 26,000, and 22,000, along with a large smear between 36,000 and 42,000 mol wt. Two of these bands (250,000 and 22,000 mol wt) had the same molecular weights as the bands that persisted or increased in intensity after prolonged labeling in vitro without NaF. If the in vivo-labeled cilia were prepared in the absence of NaF, almost all of the phosphopolypeptide bands, with the exception of the four intensely labeled ones, were lost (Fig. 9 *b*).

In Vitro Protein Phosphorylation in Cilia from Mutants

Using the known characteristics of the endogenous phosphoproteins of wild-type cells, we screened behavioral mutants of *Paramecium tetraurelia* cultured in Cerophyl medium to look for differences in phosphorylation. We found no reproducible, qualitative differences in the major phosphoproteins, the effect of cyclic GMP and cyclic AMP or the presence of the phosphatase in the following mutants: Pawn A (d4-94) (three ex-
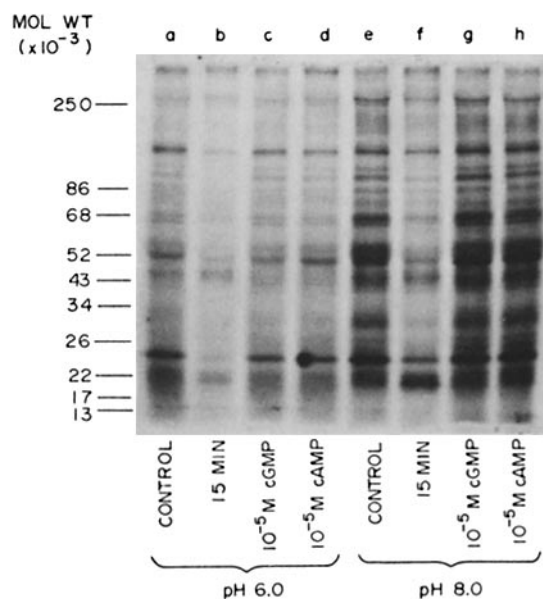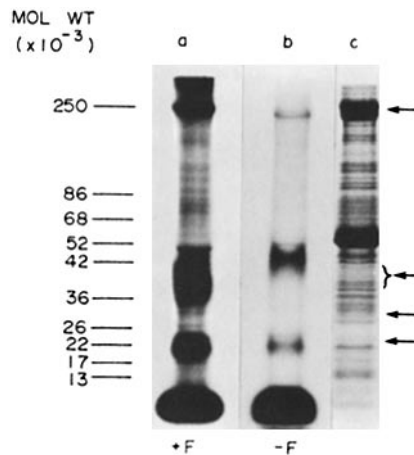

FIGURE 9 In vivo ciliary phosphoproteins. Cilia (100 µg) were from cells grown in Cerophyl on ^{32}P-labeled *E. aerogenes*. A fluorescent screen was used to intensify the image. The very intense band at the bottom of each track is probably phospholipid. (*a*) + 5 mM NaF; (*b*) without NaF. The autoradiograph shown is from one of four experiments with similar results; *c*) Coomassie-stained track; arrows indicate the four regions most heavily labeled in vivo.

periments); Pawn B (d4-95) (four experiments); fna^P (d4-149) (five experiments); Paranoiac C (d4-150) (two experiments); and fna (d4-91) (three experiments).

Cyclic GMP (10^{-5} M) stimulated the phosphorylation of the same polypeptides in wild-type and Paranoiac A (d4-90) cilia (three experiments). However, if histone was used as an exogenous substrate, the protein kinase activity of the Paranoiac A cilia at pH 6.0 was stimulated only 1.7-fold (±0.24 SD) by 10^{-5} M cyclic GMP. In contrast, wild-type ciliary protein kinase is stimulated 2.5-fold (± 0.26 SD) under identical conditions (14). This difference is highly significant by Student's *t* test (*P* < 0.001; *n* = 4 for Paranoiac A; *n* = 7 for wild type). The degree of stimulation by 10^{-5} M cyclic AMP was the same in Paranoiac A (mean ± SD = 1.6 ± 0.20, *n* = 4) and wild type (mean ± SD = 1.8 ± 0.33, *n* = 10) (14) at pH 6.0 with histone as an exogenous substrate. For the endogenous substrates, the stimulation of phosphorylation of the 31,000 mol wt polypeptide by cyclic GMP at pH 8.0 was the most significant difference in the wild type and Paranoiac A. Stimulation of phosphorylation by cyclic GMP and cyclic AMP of the 320,000 mol wt polypeptide at pH 8.0 may also be significantly different (Table I [*c* and *f*]).

DISCUSSION

We have attempted to determine whether protein phosphorylation plays a role in ciliary regulation of *Paramecium tetraurelia* by studying the characteristics of the phosphoproteins labeled in vitro by the endogenous protein kinases of the cilia (14). We established several minimum criteria to determine whether phosphorylation and dephosphorylation of proteins could regulate either ion flux across the ciliary membrane or the direction and speed of the ciliary beat: (*a*) the proteins labeled in vitro must correspond to the proteins labeled in vivo; (*b*) the phosphorylation must be reversible (i.e., both protein kinase and phosphoprotein phosphatase must be active on the substrates); (*c*) the phosphorylation must be subject to specific control by physiologically relevant changes in the cilia; (*d*) the protein kinases must be compartmentalized and/or show substrate specificities; and (*e*) mutations in protein phosphorylation should affect ion fluxes across the membrane or the

FIGURE 8 In vitro protein phosphorylation in cilia from axenically grown cells. Cilia (100 µg) from cells grown axenically in the Soldo crude medium were labeled in vitro for 15 s (unless noted). (*a*) pH 6.0, control; (*b*) pH 6.0, 15 min; (*c*) pH 6.0, 10^{-5} M cyclic GMP; (*d*) pH 6.0, 10^{-5} M cyclic AMP; (*e*) pH 8.0, control; (*f*) pH 8.0, 15 min; (*g*) pH 8.0, 10^{-5} M cyclic GMP; (*h*) pH 8.0, 10^{-5} M cyclic AMP. The autoradiograph shown is from one of five experiments with similar results.

mechanics of ciliary reversal and should thus be detectable in behavioral mutants of *P. tetraurelia*. These criteria would allow us to pinpoint specific polypeptides of potential interest for future studies.

Approximately one third of the 60–80 ciliary polypeptides visible by Coomassie staining on SDS polyacrylamide gels were phosphorylated in vitro. The relative intensity of the autoradiograph of each phosphopolypeptide band was qualitatively consistent from experiment to experiment. (Over 70 individual preparations of wild-type cilia were used.) A corresponding set of phosphopolypeptides was labeled in vivo (Fig. 9a) and was very susceptible to dephosphorylation (Fig. 9b). It is conceivable that some or all of the "in vivo" labeling is an artifact resulting from protein kinase activity during the isolation of cilia. We consider this very unlikely; the work-up is carried out at 4°C, and detached cilia, which do not form closed structures, probably lose most of their ATP during the washing procedure.

In the in vitro assays, cilia were preincubated without ATP to allow the endogenous phosphoprotein phosphatase (14) to remove any susceptible phosphate groups before the assays were initiated; ciliary polypeptides could then be rapidly phosphorylated by the endogenous protein kinases and again dephosphorylated. Turnover of the phosphate groups is an important requirement for a regulatory role for phosphorylation. The rate of phosphorylation suggested by these experiments is probably an underestimate, because the concentration of ATP used is below that needed for maximum activity of the kinases (14). This may also explain why the estimates of the stoichiometry of phosphorylation (moles P/mole peptide) are low when calculated from values for 15 s incubations. Other enzymes, such as adenylate cyclase and ATPases (29, 30; L. Riddle, A. Levin, J. Rauh, and D. L. Nelson, unpublished data) may also be competing for available ATP. Similar proteins were labeled if 1 mM ATP was used in the assays, but, in order to maintain a high enough specific activity to detect the label, the amount of [γ-^{32}P]ATP used had to be increased to a level that was impractical for routine use (data not shown).

The phosphorylation of specific polypeptides was enhanced by cyclic GMP or cyclic AMP, which suggests a method of control analogous to that in other more thoroughly studied systems (5, 6, 31). We previously reported that the phosphorylation of protamine sulfate by the ciliary protein kinase at pH 8.0 with a higher concentration of ATP (1 mM) was slightly inhibited by cyclic GMP or cyclic AMP (14). It is possible that, as in the case of rabbit erythrocyte protein kinase (32), protamine dissociates the catalytic and regulatory subunits and therefore indirectly reduces the apparent cyclic nucleotide dependence. The higher concentration of ATP may have increased the ATP(Mg^{2+})-induced inhibition of cyclic AMP binding to the regulatory subunit (33, 34).

Paramecium cells microinjected with cyclic GMP, cyclic AMP, or 3-isobutyl-1-methylxanthine lose the ability to reverse their ciliary beat and to swim backwards in the presence of ions that normally cause backward swimming in wild-type cells. Further studies and additional controls in the microinjection experiments are now in progress (C. Kung, and N. Haga, University of Wisconsin–Madison, personal communication). This evidence indirectly supports a role for cyclic nucleotide-dependent protein phosphorylation in inhibiting ciliary reversal. Indomethacin inhibits the cyclic AMP–dependent stimulation of phosphorylation of specific proteins, but the significance of this observation has not yet been determined. Addition of indomethacin to the test solution had no obvious effect on *Parmaceium* behavior in vivo (R. M. Lewis, and D. L. Nelson, unpublished observations), but this may be attributable to low permeability of *Paramecium* membranes to indomethacin. The behavioral tests used would not detect quantitative changes in the frequency or duration of ciliary reversal.

Although the ions that alter *Paramecium* behavior in vivo (i.e., Ca^{++}, K$^+$, Na$^+$, Ba^{++}) have no direct effect on phosphorylation, they may affect the enzymes that regulate the local levels of cyclic nucleotides in the cilia. The activity of the guanylate cyclase in *Paramecium* cilia is inhibited by Ca^{++} in the presence of Mg^{++} or Mn^{++} (16).

The data on phosphoproteins of the subciliary fractions (Figs. 2 and 4) reveal which of the membrane polypeptides are labeled by the protein kinase(s) associated with the membrane and which of the axonemal polypeptides are labeled by the protein kinase(s) associated with the axoneme. Some substrate specificity might occur in cilia by compartmentalization, if the membrane substrates were more accessible to membrane kinases and the axonemal substrates were more closely associated with the axonemal kinases. We attempted to determine which axonemal proteins were phosphorylated by the membrane kinases (and vice versa) by first labeling whole cilia in vitro and then isolating membranes and axonemes. This attempt was unsuccessful because we could not completely inhibit dephosphorylation by the endogenous phosphoprotein phosphatase during the subsequent steps in the fractionation (data not shown).

The phosphopolypeptide pattern of cilia obtained from axenically grown cells was similar to that of cells grown on bacteria (Fig. 8), eliminating the possibility that the protein kinases, phosphoprotein phosphatases, and phosphoprotein substrates are of bacterial origin (35). The reason for the lack of response to cyclic nucleotides is not immediately obvious. It seems unlikely that the bacteria could affect the assay, because they represent only 0.001% of the total protein in each assay. Protein kinases from the cilia of axenically grown cells are sensitive to cyclic nucleotides if exogenous substrates are used (14, 15). These exogenous substrates, however, are added in large excess over the amount of endogenous substrate present and may therefore mask differences in substrate specificity of kinases from axenically and bacteria-grown cells. Other differences between axenically grown and bacteria-grown cells have been detected: axenic cultures have slower growth rates and the cells' mitochondria are structurally and chemically different (36), and the lipid composition of *Paramecium* varies with the growth medium used (37–39). We chose to characterize the endogenous substrates of cilia from cells grown in bacterized Cerophyl so that our biochemical studies would be directly comparable with previous behavioral, electrophysiological, and genetic studies.

Initial screening of several behavioral mutants of *Paramecium* revealed that the major ciliary phosphoproteins, the phosphoprotein phosphatase, and the cyclic nucleotide effects were not qualitatively altered. The ciliary protein kinase of the Paranoiac A mutant has a lower sensitivity to stimulation by cyclic GMP than the wild-type ciliary kinase. The Paranoiac A mutation would be expected to result in a minor, quantitative change rather than an all-or-nothing difference, because the behavior of Paranoiac A is actually an exaggeration of normal wild-type behavior. Schultz and Jantzen (15) have resolved three protein kinases from *Paramecium* cilia; one of these is cyclic GMP–dependent and may correspond to the activity

that is altered in the Paranoiac A mutant. Piperno et al. (40, 41) have very recently shown that several peptides in the flagellum of *Chlamydomonas reinhardtii* are labeled during a short pulse of $^{32}P_i$. The role of phosphorylation is also unknown in this system; paralyzed mutants of *Chlamydomonas* show the same phosphorylation pattern as wild type.

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